HIGH PRESSURE NEBULIZATION AS APPLICATION ROUTE FOR THE PERITONEAL ADMINISTRATION OF SIRNA COMPLEXES

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ABSTRACT

Peritoneal Carcinomatosis (PC) is a severe form of cancer in the abdomen, currently treated with cytoreductive surgery (CRS) and intravenous (IV) chemotherapy. Recently, nebulization has been proposed as a less invasive strategy for the local delivery of chemotherapeutic drugs. Also, RNA interference has been considered as a potential therapeutic approach for treatment of cancer. In this study, Lipofectamine® RNAiMAX/siRNA complexes and cyclodextrine/ siRNA complexes were evaluated before and after nebulization. Nebulization of the siRNA complexes did not significantly lower transfection efficiency when compared to non-nebulized complexes. After incubation in ascites fluid, however, the cyclodextrine/ siRNA complexes showed a drastic decrease in transfection efficiency. For the Lipofectamine® RNAiMAX/ siRNA complexes, this decrease was less pronounced. We conclude that nebulization is an interesting technique to distribute siRNA complexes into the peritoneal cavity, providing the complexes are stable in ascites fluid which might be present in the peritoneal cavity.

INTRODUCTION

Peritoneal Carcinomatosis (PC) is a severe form of cancer with a complicated pathogenesis and poor prognosis[1,2]. It often results from a primary tumor of organs within the abdomen, such as the ovaries and colon[1]. In the process of dissemination, cancer cells first detach from the primary tumor. Then, they attach to the peritoneal mesothelium followed by infiltration into the interstitial space, proliferation and the development of new vasculature[3].

Current treatment of peritoneal carcinomatosis implies cytoreductive surgery to remove all visible tumor cells, followed by intravenous (IV) chemotherapy to eliminate the remaining tumor nodules in the peritoneal cavity[4,5]. Alternatively, intraperitoneal chemotherapy is being used, through which drugs are locally administered into the peritoneal cavity to enhance drug exposure to the tumor metastases and lower the systemic toxicity[6,7]. Also, higher drug concentrations are expected to reach avascular tumors in comparison with IV administration[8,9]. Recently, pressurized intraperitoneal aerosol chemotherapy (PIPAC) is used as a novel drug delivery method in the treatment of peritoneal carcinomatosis. In PIPAC, chemotherapeutic drugs are nebulized throughout the peritoneal cavity by applying a high pressure (~20 bars), ensuring a homogenous distribution of small aerosol particles of the drug[10,11]. PIPAC is thought to enhance the therapeutic index by local administration into the abdomen, is a minimally invasive technique and has the ability to overcome the elevated interstitial fluid pressure in tumor nodules by applying high-pressure[7,10,11].

Currently, PIPAC is evaluated in the clinic for nebulization of chemotherapeutics such as cisplatin and oxaliplatin. Apart from chemotherapeutics, however, also the use of nucleic acids has the potential to treat peritoneal carcinomatosis[12]. In RNA interference (RNAi), small interfering RNAs (siRNAs) can be used to downregulate cancer-associated genes. The short double stranded RNA molecules composed of 21-23 nucleotides result in the cleavage of

specific mRNA molecules, leading to a decrease in protein expression[13]. In contrary to conventional chemotherapy, a specific targeting of tumor cells can be obtained[14]. In order to knockdown a specific gene, siRNAs should be delivered into the cytoplasm of the cells[15]. Nevertheless, naked nucleic acids are relatively unstable, sensitive to enzymatic degradation by nucleases and quickly eliminated through filtration by the kidneys[16]. Additionally, due to the hydrophilic nature and anionic charge, almost no intracellular uptake occurs due to the poor interaction with the cellular membrane[12,16].

To enhance the transfection efficiency, appropriate delivery vehicles are needed to deliver siRNA into the cells[12,16]. Especially nanoparticles have gained interest in the field of gene delivery since they can facilitate the uptake of nucleic acids into cancer cells and provide the possibility of targeting specific cell types. In general, positively charged carriers interact with the negatively charged nucleic acids, leading to the formation of complexes based on electrostatic interactions. Both lipid-based carriers and polymer-based carriers are used, leading to the spontaneous formation of respectively lipoplexes or polyplexes[17].

Nanoparticles encounter several extracellular and intracellular barriers before siRNA is efficiently delivered into the cytoplasm where it can exert its function[18]. Extracellularly, the nanoparticles have to ensure that the siRNA remains undamaged when circulating through the body. When developing nanocarriers for intraperitoneal delivery, stability in the extracellular fluids is an important requisite[19]. Proteins in the blood and ascites fluid may, however, lead to a premature siRNA-release and/or aggregation of the particles[4,19]. When nanoparticles interact with the cell membrane they are mostly taken up by endocytosis. Then, endosomal escape should occur to avoid lysosomal degradation and release the siRNA into the cytoplasm where it can exert its function. Clearly, all these barriers might influence the biological activity of siRNA containing nanoparticles.

In this work, we focused on two different nanocarriers, namely the lipid-based Lipofectamine® RNAiMAX and a polycationic amphiphilic cyclodextrin termed as ADM70 (CD ADM70). Cyclodextrins (CDs) are a family of cyclic oligosaccharides and composed of α-(1→4)- linked d-glucopyranose units[20-22]. The cyclodextrin used in this study belongs to the family of polycationic amphiphilic cyclodextrins (paCDs) and consists of thiourea segments, polycationic clusters and lipophilic tails as described before[21,23,24]. The thiourea segments together with the polycationic clusters are used to efficiently complex nucleic acids such as siRNA[21,23,24]. The resulting complex is positively charged, facilitating the interaction with the negatively charged cell membrane and cellular uptake[24]. Using both carriers, we evaluated whether high pressure nebulization of the resulting siRNA containing nanoparticles has an effect on their biological activity. Therefore, the transfection efficiency of siRNA complexes was evaluated on a SKOV-3 cell line before and after nebulization. Furthermore, the influence of ascites fluid on the efficiency of the siRNA complexes was studied after a 1-hour incubation period, to evaluate the stability of the complexes in this biofluid that might be present in patients with peritoneal carcinomatosis[25].

EXPERIMENTAL SECTION

Materials

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma Aldrich (Bornem, Belgium). Penicillin-Streptomycin (5000 U/ml), Lipofectamine® RNAiMAX Transfection Reagent, L-Glutamine (200 mM), 0.25% Trypsin-EDTA (1X) Phenol Red, McCoy's 5A (Modified) and Opti-MEM™ were purchased from Invitrogen (Merelbeke, Belgium). The cyclodextrin ADM70 was synthesized as described elsewhere[24]. Luciferase Assay Substrate was purchased from Promega (Madison, WI, USA). Fetal Bovine Serum (FBS) was purchased from HyClone® Thermo Scientific (Cramlington, UK). Passive Lysis Buffer and Luciferase Assay Kit were purchased from (Promega, Leiden, Netherlands). The ascites fluid was obtained from a peritoneal carcinomatosis patient at the Ghent University Hospital, as approved by the ethical committee of the Ghent University Hospital (no. 2013/589). Negative-control siRNA (siNEG) and luciferase siRNA (siLuc) were purchased from Eurogentec (Searing, Belgium) and diluted in 250 µL RNAse free water to a final concentration of 20 µM.

Complex preparation and characterization

The RNAiMAX/siRNA complexes were prepared at different RNAiMAX (µl) to siRNA (pmol) ratios as follows: negative control siRNA or luciferase siRNA was diluted with Opti-MEM™ (solution A). Then, the appropriate volume of RNAiMAX was added to Opti-MEM™ (solution B) and complexes were obtained by mixing equal volumes of solution A and B, followed by incubation at room temperature for 30 minutes. Ratios are expressed as volume of RNAiMAX used to complex 10 pmol siRNA, with N/P ratio 3 being recommended by the manufacturer. To prepare cyclodextrin/siRNA complexes at different N/P ratios, equal volumes

of a cyclodextrin-solution in HEPES buffer was added to a solution containing 1 pmol/µl of negative control siRNA or luciferase siRNA, and incubated for 30 minutes at room temperature. Different N/P ratios were made by changing the concentration of the cyclodextrin-solution, with a maximum of 0.42 mM to obtain N/P ratio 10. Throughout this paper, the cyclodextrins will be termed as ADM70.

DLS measurements were performed using solely the firefly luciferase siRNA. RNAiMAX/siRNA complexes were prepared at N/P ratios of 2, 3, 4, 6 and 8 with a total volume of 100 µL and at a final concentration of 0.5 µM siRNA. ADM70/siRNA complexes were prepared at N/P ratios of 4, 6, 8 and 10 and at a final siRNA concentration of 0.5 µM. 800 µL of HEPES buffer was added to the complexes and the suspension was transferred to the zeta cell (Malvern cells, UK) with a 1 mL syringe (BD Plastipak™, USA). All samples were prepared in a dust free laminar flow (LAF) and measurements were performed using a Zetasizer Nano-ZS **(**Malvern instruments, Worcestershire, UK) at 25°C. DLS measurements of complexes prepared in Opti-MEM™ or biological fluids are not possible since proteins and other macromolecules can interfere with the measurements[19]

Agarose gel electrophoresis

Gel electrophoresis was used to test at which N/P ratios stable complexes are formed. 1 g agarose (Invitrogen by Life Technologies™, Carlsbad, USA) was dissolved in 80 mL of heated deionized water. Next, the solution was diluted to a final volume of 100 mL with cold deionized water. 10 µL of gelred (Biotium, Hayward, USA) was added to the solution. The solution was allowed to cool to 50°C and poured into the gel holder. Samples for gel electrophoresis were prepared using firefly luciferase siRNA. RNAiMAX/siRNA and ADM70/siRNA complexes had a final concentration of 0.5 pmol/µl siRNA and N/P ratios of 0; 0.5; 1; 2; 3; 4; 6; 8 and 0; 0.5; 1; 2; 4; 6, 8, 10 respectively. Then, 5 µL of loading buffer was

added to 20 µl of each sample. When stability of complexes in ascites fluid was evaluated, an incubation time of 20 minutes in 10 µL ascites was incorporated before addition of the loading buffer.

Cell culture and transfection experiments

In vitro transfections were performed in the human ovarian cancer cell line SKOV-3 which stably expresses firefly luciferase. Cells were cultured in McCoy's 5A medium supplemented with FBS, Penicillin-Streptomycin and L-Glutamine. Cells were cultured until 80% to 90% confluency and detached from tissue culture dishes with 0.25% trypsin. Cells were maintained in an incubator at 37°C in a humidified atmosphere with 5% CO_2. For the transfection experiments, SKOV3 cells were plated 24 h prior to the experiment into 24-well plates at 50000 cells/well. Subsequently, each well was provided with the appropriate amount of complex solution in 500 µl Opti-MEM^{TM}, corresponding to 1, 2, 5, 10, 25 or 50 pmol of siRNA per well. After 4 h incubation, the transfection medium was replaced by fresh culture medium. Each formulation (containing siRNA duplex firefly (siRNA luc)) was compared with its own control (containing siRNA pGL3 luciferase control (siRNA ctrl)). After 24 hours incubation, cells were lysed with Passive Lysis Buffer and analyzed for firefly luciferase expression using the luciferase assay kit (Promega). The luminescence (Relative Light Units, RLU) was measured using a GloMax Luminometer (Promega). The percentage of luciferase downregulation was determined by the following equation: % transfection = 100 – (100 x RLU_{luc}/RLU_{ctrl}), where RLU_{ctrl} is the mean for control siRNA and RLU_{luc} is the mean for luciferase siRNA.

Complex nebulization

To evaluate the effect of aerosolization on the physicochemical properties and transfection efficiency of the complexes, 300 µl of complexes (containing 0.5 pmol/µl siRNA) were

aerosolized using a Microsprayer® Aerosolizer in combination with a high-pressure syringe (Penn-Century, Wyndmoor, USA) as described before[26]. Aerosolized complexes were collected in Eppendorf vials (typically > 90% recovery). Then, the nebulized complex solutions were used for size and zeta potential measurements and for transfection experiments as described above. All transfection experiments were carried out in triplicate for each treatment to allow calculation of the standard deviation.

Complex incubation in ascites fluid

RNAiMAX/siRNA and cyclodextrin/siRNA complexes were prepared at a concentration of 0.5 and 2.5 pmol/µl siRNA respectively. For the transfection experiments with an amount of 5 pmol and 25 pmol siRNA/well, 50 µL of these complex solutions was added to 50 µL ascites fluid. After 1-hour incubation at 37°C, 100 µL of the incubated solution was added to 400 µL Opti-MEM™ in the well. For the RNAiMAX/siRNA and cyclodextrin/siRNA transfection with an amount of 10 pmol and 50 pmol siRNA respectively, 100 µL of the complex solution was added to 100 µL ascites. After 1-hour incubation at 37°C, the 200 µl mixture was added to 600 µL Opti-MEM™ in the well.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 7 software, by using an analysis of variance (ANOVA) followed by the Sĭdák Multiple Comparisons test with an α-value set at 0.05. For each formulation, the transfection efficiency before and after nebulization was compared.

RESULTS AND DISCUSSION

Complex formation and stability in ascites fluid.

As a first step in the characterization of siRNA containing complexes, a gel retardation assay was performed to determine the optimal N/P ratio for siRNA encapsulation. Also, the stability of the complexes was evaluated upon incubation in ascites fluid.

Figure 1A depicts the complexation efficiency of RNAiMAX/siRNA complexes at different N/P ratios immediately after preparation in Opti-MEM™. For the N/P ratio of 0, solely siRNA is loaded into the well. For N/P ratios 0.5 and 1, free siRNA (asterisk) can be detected on the gel, which is attributed to the low amount of Lipofectamine® RNAiMAX used to prepare these solutions. Starting from N/P ratio of 2, free siRNA is no longer detected and complexes appear in the wells of the gel (open circle). It seems that the amount of positive charges is sufficient to form electrostatic interactions with the majority of negatively charged siRNA starting from N/P ratio 2. When the N/P ratio is increased even more, the intensity of the complexes in the wells seems to increase. We hypothesize that the siRNA appears more bright as the fixed amount of siRNA is able to spread over a larger number of RNAiMAX liposomes, increasing their accessibility to the Gelred used to detect the siRNA. Figure 1B shows the complexation efficiency of the ADM70/siRNA complexes at different N/P ratios after preparation in HEPES buffer. In contrast to the Lipofectamine® RNAiMAX complexes, full complexation of siRNA is only visible at N/P ratio 6 while at N/P ratio 4 some complexes appear but also a smear of free siRNA is still noticeable on the gel. Upon increasing the amount of positively charged ADM70, however, more stable complexes are formed with the ability to fully complex siRNA.

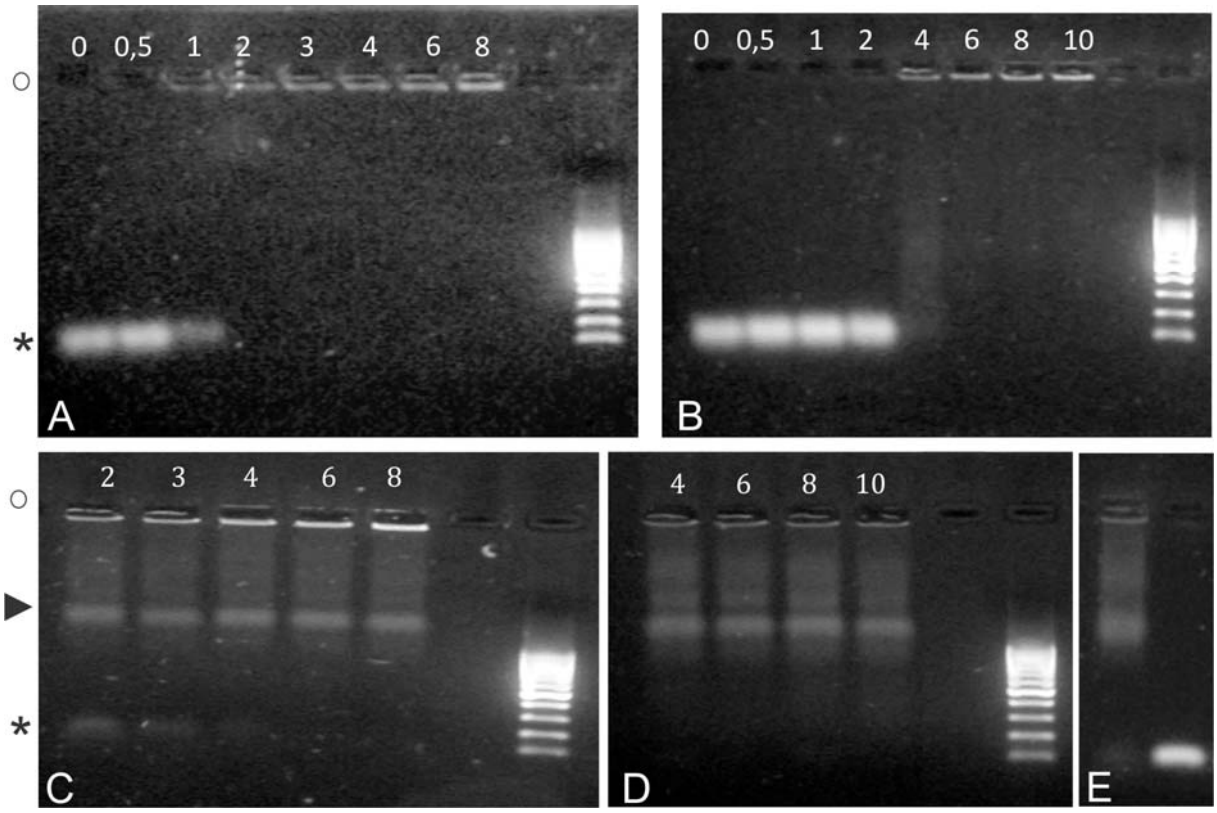

Figure 1. Agarose gel electrophoresis of (A) RNAiMAX/siRNA complexes and (B) ADM70/siRNA complexes prepared at different N/P ratios as depicted above each well. The final amount was 10 pmol siRNA/well. A 100 basepair (bp) ladder is positioned in the last well. (C) and (D) represent the RNAiMAX/siRNA and ADM70/siRNA complexes following incubation in ascites fluid. (E) represent ascites fluid alone (first lane) and free siRNA (second lane). Open circle, arrowhead and asterisk point to the position of complexes, ascites fluid and free siRNA respectively.

One of the objectives of this work is to study the stability and biological activity of the complexes in a relevant biological fluid for PC. Therefore, the complexation efficiency of the siRNA complexes was determined in ascites fluid obtained from a peritoneal carcinomatosis patient. It has already been suggested that a protein-rich environment can alter the identity of nanoparticles, leading to cargo release, aggregation, inhibition of uptake and decrease of biological activity[19,27]. When ascites fluid results in the release of siRNA from the complexes, this should be noticeable by the re-appearance of free siRNA in the gel. Figure 1C shows that free siRNA bands were observed for Lipofectamine® RNAiMAX complexes prepared with

N/P ratios 2, 3 and 4. A possible explanation for this siRNA release is the competition between siRNA and negatively charged proteins in the ascites fluid for binding to the carrier[4,19]. However, this is highly likely not a full release since clear staining in the wells is still visible and the band intensities are much less than the free siRNA control (Figure 1E). At N/P ratio of 8 no free siRNA bands are present, implying a greater stability in comparison with the other N/P ratios. When ADM70/siRNA complexes are incubated in ascites fluid, no free siRNA is detected while complexes are seen in the wells (Figure 1D). It should be noted that the ascites fluid itself is also detected on the gel, but does not interfere with the interpretation of the siRNA band (Figure 1C-E, arrowhead). It seems that, following incubation in ascites, cyclodextrin-complexes are more stable in terms of siRNA release in comparison with Lipofectamine® RNAiMAX complexes.

Effect of nebulization on the physicochemical properties of siRNA complexes

Table 1 depicts the average diameter of RNAiMAX/siRNA complexes before and after nebulization, using N/P ratios that lead to good siRNA complexation as determined above. For each of the studied N/P ratios, a minor increase in the average diameter was measured. Therefore, it seems that complexes are not majorly influenced by the nebulization process as no severe aggregation was seen for all the RNAiMAX/siRNA complexes. Importantly, the complexes remain in a nanometer size range following nebulization, which enables them to cross biological membranes and deliver their cargo into the desired cells[28]. Interestingly, nebulization also did not significantly affect the polydispersity index (PDI) of the complexes, providing further evidence that nebulization did not result in destabilization of the complexes.

Table 1. Size measurements at different N/P ratios of the RNAiMAX/siRNA complexes prepared at a final amount of 50 pmol siRNA. The numbers in the table represent the hydrodynamic diameter (nm) ± SD. N=3.

N/P	Non-Nebulized[a]	PDI[b]	Nebulized[a]	PDI[b]
2	179 ± 8	0.3	298 ± 5	0.5
3	141 ± 1	0.4	193 ± 8	0.4
4	152 ± 3	0.4	234 ± 5	0.4
6	143 ± 7	0.3	216 ± 6	0.4
8	121 ± 9	0.5	131 ± 7	0.4

[a] Size in nanometer (nm) [b] Polydispersity index

When ADM70/siRNA complexes were nebulized (Table 2), a more drastic effect was seen on the complex characteristics. Before nebulization, a decrease in size is visible for the complexes when the N/P ratio increases. It seems that a higher amount of ADM70, relative to the amount of siRNA, enables the formation of a smaller complexes. After nebulization, however, a substantial increase in the average diameter can be observed for the different N/P ratios. The largest increase following nebulization of complexes was seen for N/P ratio 10, resulting in micrometer sized aggregates. This size increase implies a form of instability of the ADM70 complexes that was not observed for the Lipofectamine® RNAiMAX complexes.

Table 2. Size measurements at different N/P ratios of the ADM70/siRNA complexes. The numbers in the table represent the hydrodynamic diameter (nm) ± SD. N=3.

N/P ratio	Non-Nebulized[a]	PDI[b]	Nebulized[a]	PDI[b]
4	452 ± 14	0.3	763 ± 11	0.4
6	315 ± 7	0.4	443 ± 22	0.4
8	268 ± 8	0.4	919 ± 28	0.4
10	209 ± 12	0.4	1360 ± 238	0.3

[a] Size in nanometer (nm) [b] Polydispersity index

Also when the charge of the complexes was determined, a difference can be seen before and after nebulization. Table 3 shows that for RNAiMAX/siRNA complexes, a clear trend of decrease in zeta potential can be observed for each N/P ratio. As the zetapotential becomes more negative, we hypothesize that this decrease is highly likely attributed to the rearrangement

of siRNA on the surface of the complexes, due to the friction or pressure applied during nebulization. However, it is important to notice that the complexes are still positively charged after nebulization, enabling interaction with the negatively charged cell membrane[29].

Table 3. Zeta potential measurements at different N/P ratios of the RNAiMAX/siRNA complexes. The numbers in the table represent the mean zeta potential ± SD. N=3.

N/P	Non-Nebulized[a]	Nebulized[a]
2	25 ± 3	19 ± 1
3	38 ± 1	15 ± 1
4	43 ± 2	22 ± 4
6	44 ± 1	27 ± 2
8	41 ± 1	25 ± 2

[a] Zeta Potential in millivolt (mV)

Also for ADM70/siRNA complexes, a decrease in zetapotential is observed after nebulization (Table 4). Again, we hypothesize that a rearrangement of siRNA in the cyclodextrine complexes occurs as a result of the friction or pressure applied during nebulization. It should be noted, however, that the complexes shift from a positive charge before nebulization to being negatively charged. This indicates that siRNA migrated to the surface of the cyclodextrine complexes during nebulization, possibly lowering the protection of the siRNA by the carrier. Also, the negative charge of the complexes might lower the interaction with cells and subsequent intracellular uptake. Taken together, both sort of complexes seem to be stable in a size range that enables them to cross biological membranes before and after nebulization. However, the drop in zeta potential following nebulization of the ADM70/siRNA complexes could possibly influence the transfection efficiency.

Table 4. Zeta potential measurements at different N/P ratios of the ADM70/siRNA complexes. The numbers in the table represent the mean zeta potential ± SD. N=3.

N/P ratio	Non-Nebulized[a]	Nebulized[a]
4	13 ± 9	-20 ± 4
6	26 ± 2	-26 ± 2
8	30 ± 1	-17 ± 2
10	33 ± 1	-9 ± 3

[a]Zeta potential in millivolt (mV)

Dose-dependent transfection efficiency of siRNA complexes

Initially we performed an optimization experiment to determine the suitable concentration of siRNA that induces gene silencing without causing toxicity. The N/P ratio of Lipofectamine® RNAiMAX/siRNA and ADM70/siRNA complexes was kept at 3 and 6 respectively, being the first N/P ratio that fully complexed the siRNA as determined from Figure 1. For Lipofectamine® RNAiMAX complexes, increasing siRNA concentrations from 1 to 10 pmol lead to an efficient downregulation of luciferase of around 80%. However, for the wells treated with 25 and 50 pmol of siRNA a clear decrease in the bioluminescence of the control siRNA was noticeable. This is an indication for toxicity which is probably attributed to the presence of a high concentration of siRNA or Lipofectamine® RNAiMAX. Thus RNAiMAX/siRNA complexes of 25 and 50 pmol were not considered for further experiments.

For the cells treated with negative control ADM70/siRNA complexes (Figure 2B), no clear decrease in bioluminescence is noticeable, even not for the higher siRNA concentration. This low toxicity profile of the cyclodextrines could highly likely be the result of the amphiphilic structure as well as the presence of cationic clusters and hydrogen bonding thiourea groups, as demonstrated before[21,23,30]. It should be noted, however, that the cyclodextrines only result in a high amount of luciferase downregulation starting from 25 and 50 pmol siRNA. Therefore, it seems that the cyclodextrines used in this study need a 10-fold higher concentration of siRNA, to result in comparable levels of downregulation efficiency as Lipofectamine® RNAiMAX lipoplexes.

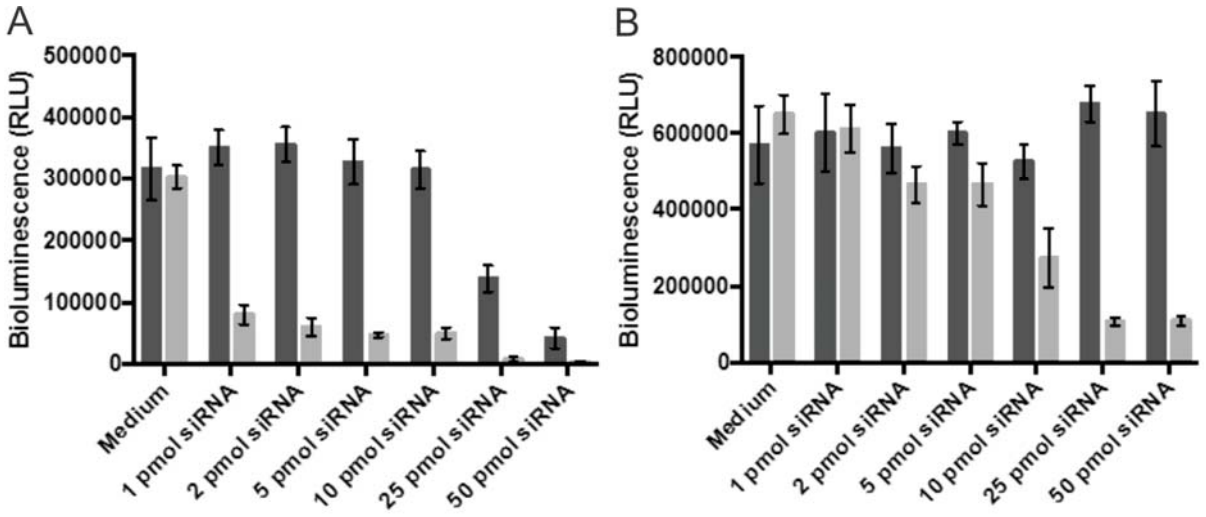

Figure 2. Bioluminescence of SKOV-3 cells treated with RNAiMAX/siRNA lipoplexes (A) and ADM70/siRNA complexes (B) containing negative Control siRNA (dark grey bars) or luciferase siRNA (light grey bars) following four hours of incubation in Opti-MEM™. Data are expressed as the average (± SD) from three independent transfection experiments.

Effect of nebulization on the transfection efficiency of siRNA complexes

Since nebulization has been proposed as a strategy to deliver chemotherapeutic drugs in the treatment of PC, we tested the transfection efficiency of the studied formulations after nebulization. DLS results have already shown an increase in hydrodynamic diameter of the lipoplexes, together with a drop in zetapotential. This raised the question if the transfection efficiency of the particles would still be sufficient after nebulization, since uptake and subsequently transfection efficiency are size and charge dependent[19].

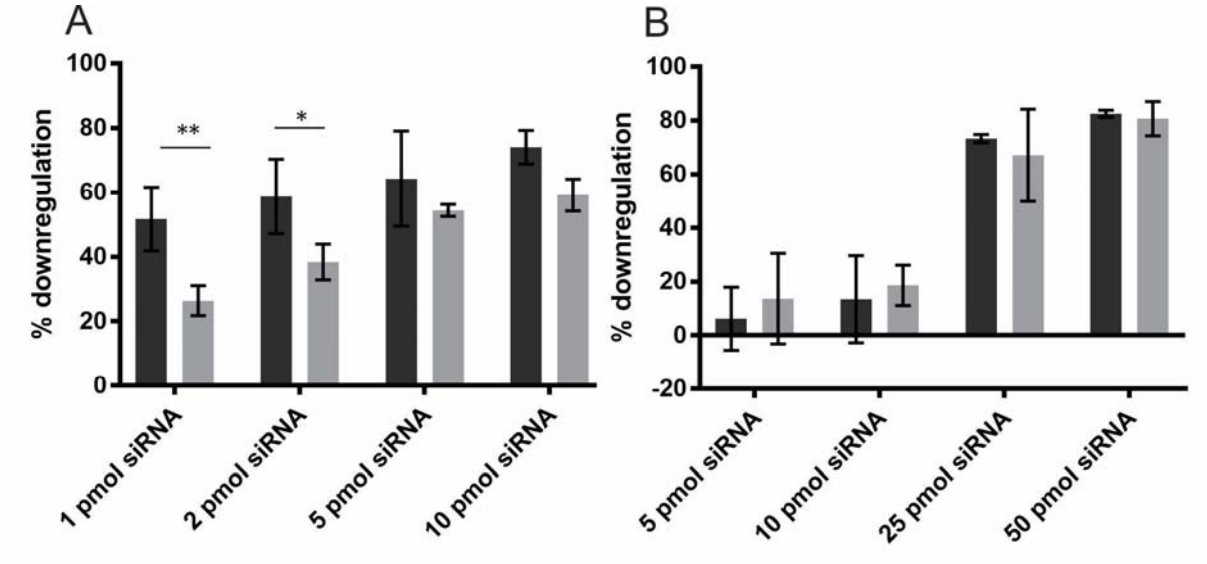

Figure 3. Percentage downregulation in SKOV-3 cells treated with by non-nebulized (dark grey bars) and nebulized (light grey bars) RNAiMAX/siRNA lipoplexes (N/P ratio 3) (A) and ADM70/siRNA complexes (N/P ratio 6) (B) following four hours of incubation in Opti-MEM™. Data are expressed as the average (± SD) from three independent transfection experiments.

Figure 3A demonstrates that a significant decrease in transfection efficiency is only observed for RNAiMAX/siRNA complexes delivering 1 pmol and 2 pmol of siRNA, whereas no significant difference was observed when 5 pmol and 10 pmol siRNA was present. Apparently, the destabilization of complexes does change transfection efficiency at lower siRNA concentrations, but can be overruled by administering a larger amount of the complexes onto the cells. For the ADM70/siRNA complexes (Figure 3B), again for the low amounts of siRNA (i.e. 5 and 10 pmol) a low transfection efficiency was noticeable, whereas for the high amounts of siRNA, the transfection is higher ranging from 65% to 80% downregulation. After nebulization, the downregulation is not statistically different, although the larger error bars demonstrate the appearance of more variability in the transfection results. This result was rather unexpected since zeta potential measurements demonstrated a negative zeta potential for these complexes after nebulization. It should be noted, however, that the complexes used for

transfection experiments were administered in Opti-MEM™ which could lead to different size and zeta potential values than presented in Table 3 and 4 respectively.

Several mechanisms can be responsible for the small loss in transfection efficiency. First of all, it is possible that the complexes lose some of their stability during nebulization. This stability is an important determinant for the overall transfection efficiency and may be hampered by premature siRNA release and aggregate formation[19,28]. As the siRNA concentration was changed by adding different volumes of the nebulized complexes, the effect of nebulization on the complex efficiency was expected to be comparable for all siRNA amounts that were delivered. If some siRNA is released during nebulization, however, it can be expected that this effect is larger for the lower amounts of siRNA (i.e. 1 and 2 pmol) as the threshold for a sufficient siRNA concentration might no longer be reached. The decrease in transfection efficiency may also be attributed to the change in structure of some siRNA molecules due to the pressure applied, as suggested for pDNA in literature[26]. Again, however, it is a possibility that when higher volumes (i.e. more complexes) are added on the cells, the amount of siRNA entering the cell and able to exert its function is still sufficient which can lead to non-significant decrease in transfection efficiency.

Effect of ascites fluid on the transfection efficiency of siRNA complexes

As mentioned earlier, one of the aims of this work is to evaluate the biological activity in a relevant biological fluid for peritoneal carcinomatosis. Therefore, the ability of the siRNA-complexes to downregulate luciferase expression, following 1 hour incubation in ascites fluid, was determined (Figure 4).

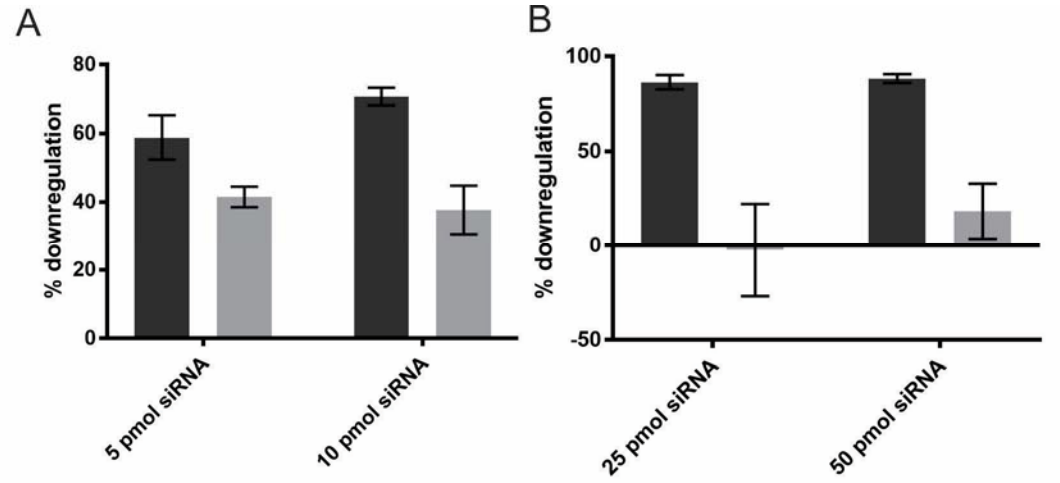

Figure 4. Percentage downregulation in SKOV-3 cells treated with RNAiMAX/siRNA lipoplexes (N/P ratio 3) (A) and ADM70/siRNA complexes (N/P ratio 6) (B) in Opti-MEM™ (dark grey bars) and following 1 hour incubation in ascites fluid (light grey bars). Data are expressed as the average (± SD) from three wells in the same transfection-experiment.

Both for RNAiMAX/siRNA complexes and ADM70/siRNA complexes, the two highest siRNA doses were chosen as these were not influenced by the nebulization of the complexes. In opti-MEM™, both type of complexes again show good transfection efficiencies. Following exposure to ascites fluid, however, a clear decrease in the transfection efficiency can be noticed for all amounts of siRNA. For the RNAiMAX/siRNA complexes, about 40% of downregulation remains. For ADM70/siRNA complexes, however, the transfection efficiency drops to less than 20% for 50 pmol of siRNA and even to 0% when 25 pmol of siRNA is delivered. The premature release of siRNA could be considered as a possible reason for the decrease in transfection efficiency, although gel electrophoresis did not show a high amount of free siRNA in ascites fluid (see Figure 1).

Another possible reason for the decrease in transfection efficiency in ascites fluid is a diminished uptake of the complexes. We have demonstrated before that incubation in ascites fluid lowers the transfection efficiency of DOTAP/DOPE siRNA complexes by lowering the

cellular uptake of the complexes[4,19]. Binding of negatively charged proteins present in ascites fluid is most likely responsible for the observed effect. Alternatively, the formed 'protein corona' can also interfere with the intracellular pathways followed by the nanoparticles, leading to non-productive siRNA delivery, as we observed for lipoplexes composed of DOTAP and cholesterol (non-published data).

Combined effect of nebulization and the presence of ascites fluid on the transfection efficiency of siRNA complexes

In the eventual therapeutic application, the complexes will first be nebulized and then come in contact with the peritoneal tumor nodules and the ascites fluid. Therefore, we tested how the combination of nebulization and the subsequent incubation in ascites fluid affected the transfection efficiency of siRNA complexes. For both RNAiMAX/siRNA complexes and ADM70/siRNA complexes, the highest siRNA dose was chosen. Figure 5 A demonstrates that for nebulized RNAiMAX/siRNA complexes, the incubation in ascites fluid (grey bars) does not further decrease the percentage of downregulation when compared to nebulized complexes administered in opti-MEM™ (black bars). For nebulized ADM70/siRNA complexes (Figure 5 B), however, the presence of ascites fluid again severely decreased the downregulation efficiency, in agreement with the effects observed on non-nebulized ADM70/siRNA complexes. The presence of ascites fluid thus strongly affects the efficiency of cyclodextrine/siRNA complexes. It should be noted, however, that large volumes of ascites fluid are only expected in patients that are in an advanced stage of peritoneal carcinomatosis. Currently, we cannot rule out the possibility that the lower amount of ascites fluid, present in patients that receive the PIPAC procedure, could have a less drastic effect on the transfection efficiency of the ADM70/siRNA complexes.

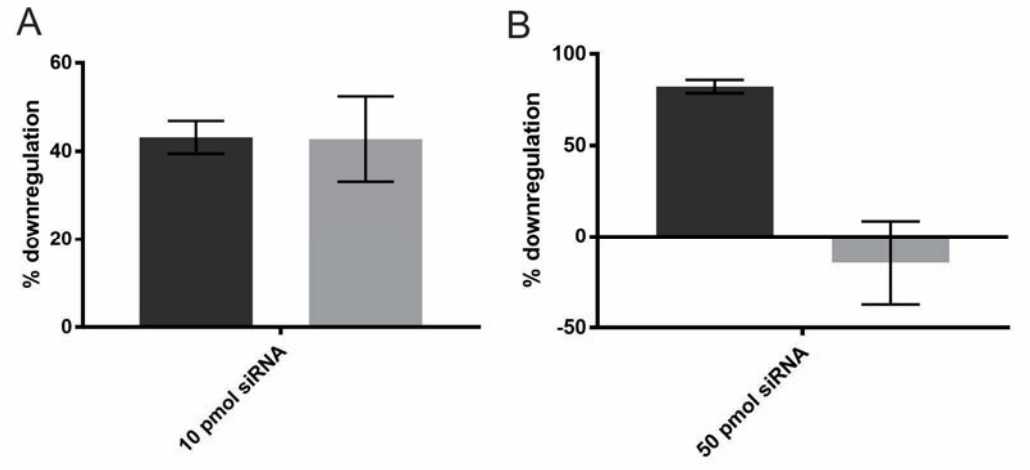

Figure 5. Percentage downregulation in SKOV-3 cells treated with nebulized RNAiMAX/siRNAlipoplexes (N/P ratio 3) (A) and nebulized ADM70/siRNA complexes (N/P ratio 6) (B) in Opti-MEM^{TM} (dark grey bars) and following 1 hour incubation in ascites fluid (light grey bars). Data are expressed as the average (± SD) from three wells in the same transfection-experiment.

CONCLUSION

Administration of siRNA complexes in the peritoneal cavity has the potential to treat peritoneal carcinomatosis. The nebulization of complexes in the peritoneal cavity has clear advantages such as a more even spreading of the complexes in the large surface area of the peritoneum. It might, however, also affect the transfection efficiency of complexes due to the high pressure applied during the procedure. We have demonstrated that both lipid-based carriers

(Lipofectamine® RNAiMAX) and polymeric cyclodextrines (ADM70) have the potential to successfully administer siRNA to ovarian cancer cells. Lipoplexes, however, reached a good transfection efficiency already at a 10 times lower siRNA dose. Upon nebulization of the complexes, a minor decrease in transfection efficiency was observed, especially for the lower siRNA concentrations. Possible reasons for this observation are the increase in size and decrease in zeta potential observed for the complexes at different N/P ratios. When higher amounts of siRNA (i.e. more complexes) were added on the cells, however, the small decrease in transfection efficiency could be overcome. The presence of ascites fluid, however, had a more drastic effect on the transfection efficiency, especially for the cyclodextrin/siRNA complexes. The loss in transfection efficiency for cyclodextrine/siRNA complexes can most likely be attributed to the formation of a protein corona around the nanoparticles, interfering with cellular uptake or the intracellular delivery pathway of siRNA. Overall, however, we conclude that nebulization is a promising administration route for siRNA complexes to the peritoneal cavity, providing the complexes remain stable in ascites fluid.

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KEYWORDS

Peritoneal carcinomatosis, nebulization, siRNA delivery, lipoplexes, cyclodextrines

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CAPTIONS TO FIGURES

Figure 1. Agarose gel electrophoresis of (A) RNAiMAX/siRNA complexes and (B) ADM70/siRNA complexes prepared at different N/P ratios as depicted above each well. The final amount was 10 pmol siRNA/well. A 100 basepair (bp) ladder is positioned in the last well. (C) and (D) represent the RNAiMAX/siRNA and ADM70/siRNA complexes following incubation in ascites fluid. (E) represent ascites fluid alone (first lane) and free siRNA (second lane). Open circle, arrowhead and asterisk point to the position of complexes, ascites fluid and free siRNA respectively.

Figure 2. Bioluminescence of SKOV-3 cells treated with RNAiMAX/siRNA lipoplexes (A) and ADM70/siRNA complexes (B) containing negative Control siRNA (dark grey bars) or luciferase siRNA (light grey bars) following four hours of incubation in Opti-MEM™. Data are expressed as the average (± SD) from three independent transfection experiments.

Figure 3. Percentage downregulation in SKOV-3 cells treated with by non-nebulized (dark grey bars) and nebulized (light grey bars) RNAiMAX/siRNA lipoplexes (N/P ratio 3) (A) and ADM70/siRNA complexes (N/P ratio 6) (B) following four hours of incubation in Opti-MEM ™. Data are expressed as the average (± SD) from three independent transfection experiments.

Figure 4. Percentage downregulation in SKOV-3 cells treated with RNAiMAX/siRNA lipoplexes (N/P ratio 3) (A) and ADM70/siRNA complexes (N/P ratio 6) (B) in Opti-MEM ™ (dark grey bars) and following 1 hour incubation in ascites fluid (light grey bars). Data are expressed as the average (± SD) from three wells in the same transfection-experiment.

Figure 5. Percentage downregulation in SKOV-3 cells treated with nebulized RNAiMAX/siRNAlipoplexes (N/P ratio 3) (A) and nebulized ADM70/siRNA complexes (N/P ratio 6) (B) in Opti-MEM™ (dark grey bars) and following 1 hour incubation in ascites fluid (light grey bars). Data are expressed as the average (± SD) from three wells in the same transfection-experiment.

FIGURES AND TABLES

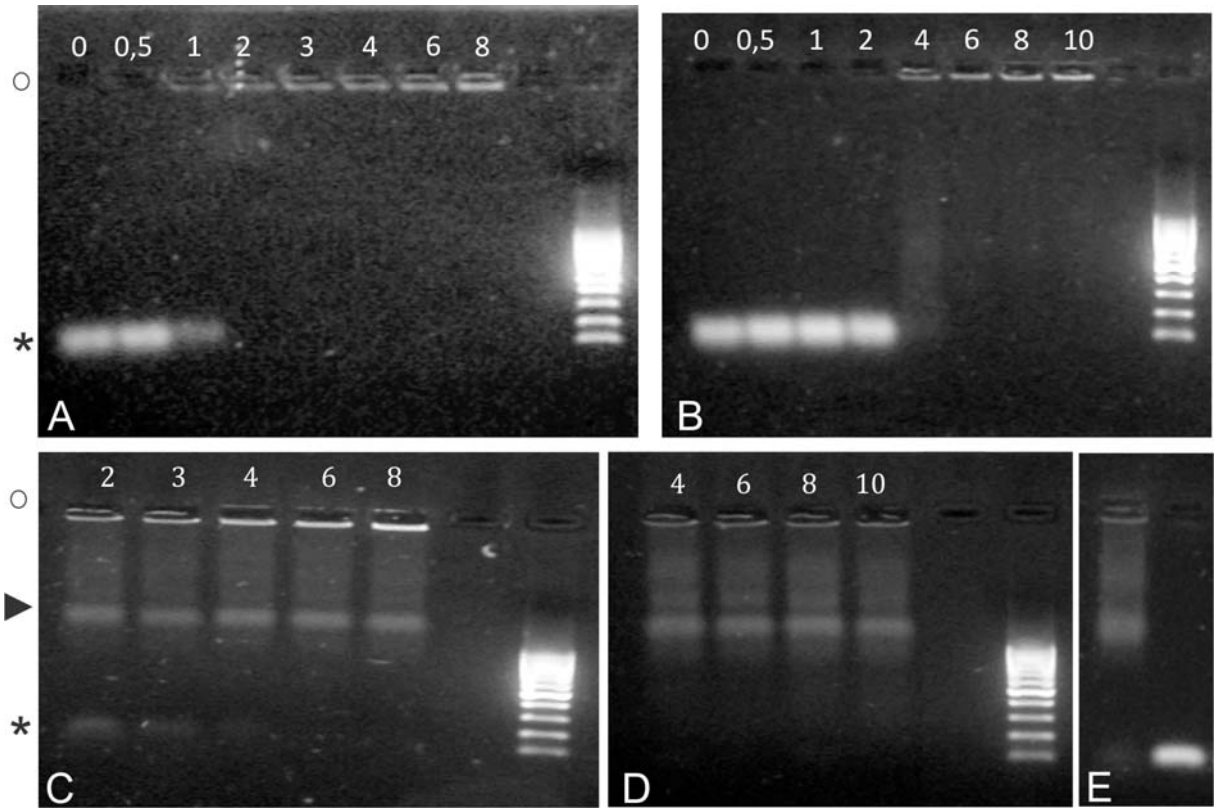

Figure 1.

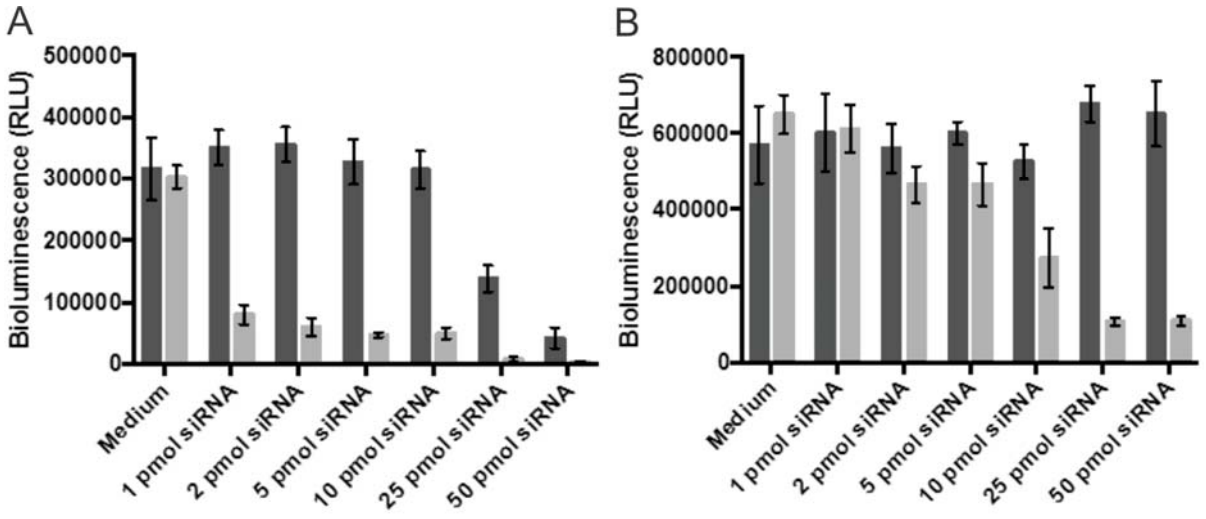
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Figure 2

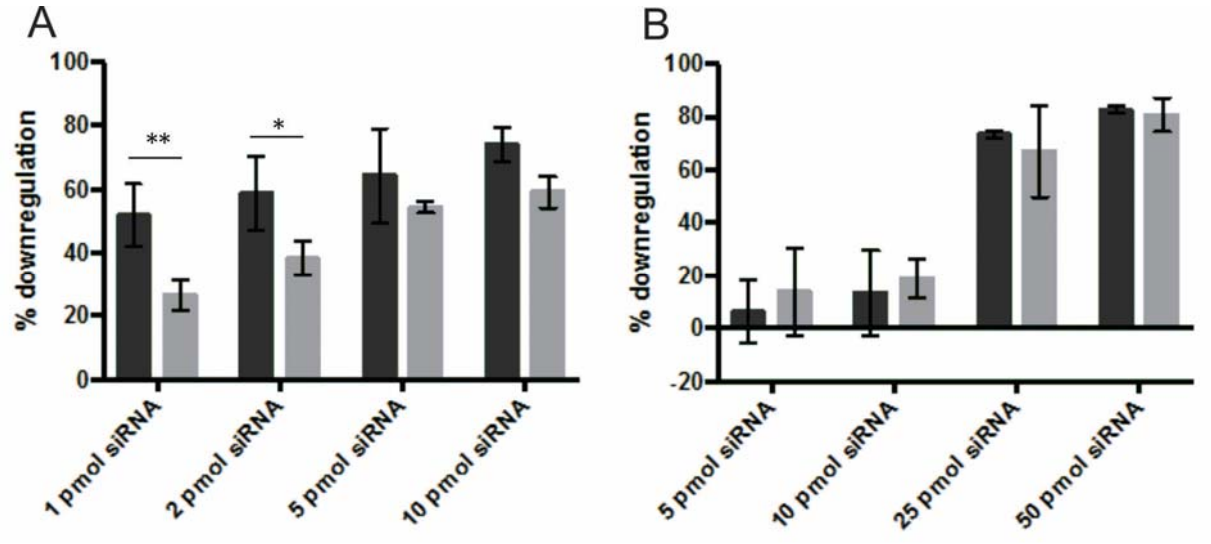
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Figure 3

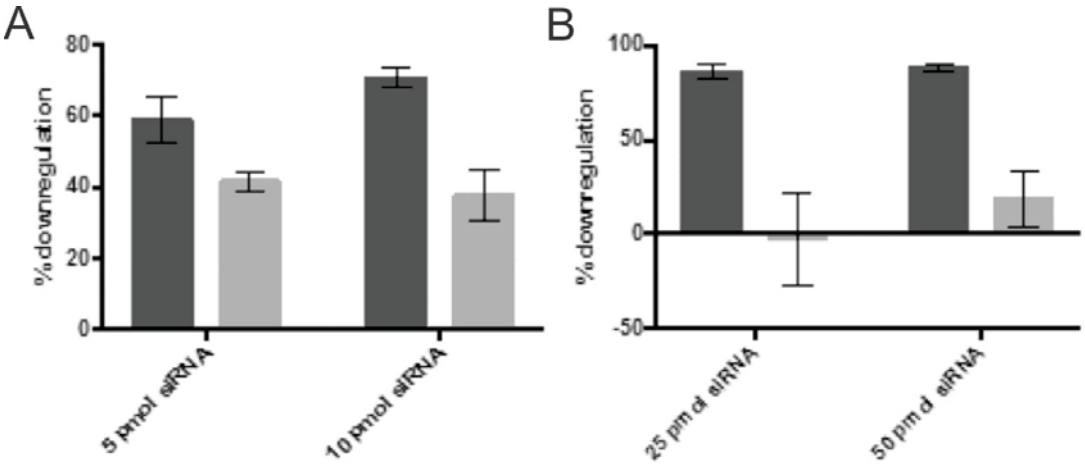

Figure 4

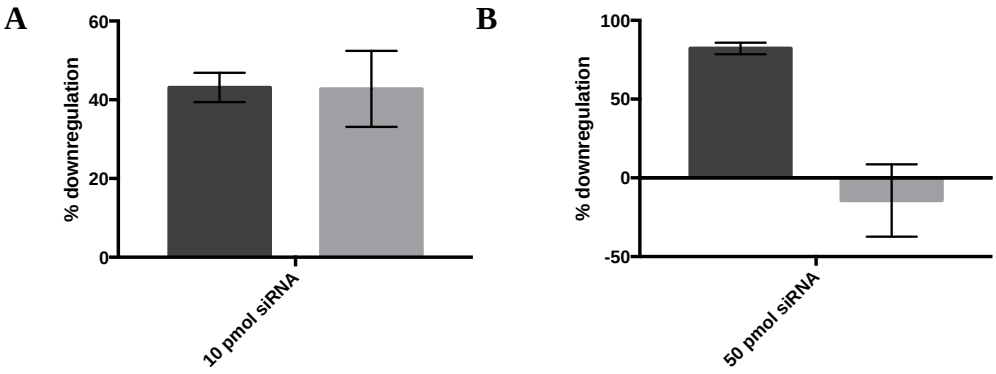

Figure 5

Table 1. Size measurements at different N/P ratios of the RNAiMAX/siRNA complexes prepared at a final amount of 50 pmol siRNA. The numbers in the table represent the hydrodynamic diameter (nm) ± SD. N=3.

N/P	Non-Nebulized[a]	PDI[b]	Nebulized[a]	PDI[b]
2	179 ± 8	0.3	298 ± 5	0.5
3	141 ± 1	0.4	193 ± 8	0.4
4	152 ± 3	0.4	234 ± 5	0.4
6	143 ± 7	0.3	216 ± 6	0.4
8	121 ± 9	0.5	131 ± 7	0.4

[a] Size in nanometer (nm) [b] Polydispersity index

Table 2. Size measurements at different N/P ratios of the ADM70/siRNA complexes. The numbers in the table represent the hydrodynamic diameter (nm) ± SD. N=3.

N/P ratio	Non-Nebulized[a]	PDI[b]	Nebulized[a]	PDI[b]
4	452 ± 14	0.3	763 ± 11	0.4
6	315 ± 7	0.4	443 ± 22	0.4
8	268 ± 8	0.4	919 ± 28	0.4
10	209 ± 12	0.4	1360 ± 238	0.3

[a] Size in nanometer (nm) [b] Polydispersity index

Table 3. Zeta potential measurements at different N/P ratios of the RNAiMAX/siRNA complexes. The numbers in the table represent the mean zeta potential ± SD. N=3.

N/P	Non-Nebulized[a]	Nebulized[a]
2	25 ± 3	19 ± 1
3	38 ± 1	15 ± 1
4	43 ± 2	22 ± 4
6	44 ± 1	27 ± 2
8	41 ± 1	25 ± 2

[a] Zeta Potential in millivolt (mV)

Table 4. Zeta potential measurements at different N/P ratios of the ADM70/siRNA complexes. The numbers in the table represent the mean zeta potential ± SD. N=3.

N/P ratio	Non-Nebulized[a]	Nebulized[a]

4	13 ± 9	-20 ± 4
6	26 ± 2	-26 ± 2
8	30 ± 1	-17 ± 2
10	33 ± 1	-9 ± 3

[a]Zeta potential in millivolt (mV)

TOC

This study demonstrates that nebulization is an interesting technique to distribute siRNA complexes into the peritoneal cavity, providing the complexes are stable in ascites fluid which might be present in the peritoneal cavity. The lipid-based RNAiMAX/siRNA complexes proved to be superior over polymeric siRNA cyclodextrin complexes at lower siRNA concentrations and in the presence of ascites fluid.